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We have cloned and sequenced the full length int-3 gene and have renamed it Notch4, since it is the fourth vertebrate Notch gene identified. The protein encoded by Notch4 is a 220 kilodalton cell surface receptor. Notch4 is a bona fide Notch receptor with 29 EGF-like repeats, 3 Notch/lin-12 repeats, and an intracellular domain with ankyrin repeats. Notch4 is expressed primarily in the adult and embryonic endothelium. Activation of Notch4, to the int-3 oncoprotein, results in a truncated receptor lacking the extracellular domain but retaining the transmembrane and intracellular domains. Notch4 receptors are membrane associated, as expected for cell surface receptors; whereas the int-3 oncoprotein resides in the nucleus. Activated int-3 can block hepatocyte growth factor induced ductal morphogenesis of an established normal human mammary epithelial cell line, TAC-2. Int-3 does not stimulate increased cell growth of TAC-2 cells. The ability of int-3 to block morphogenesis resembles its function in oncogenesis in the mammary gland.

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Int-3 Oncogene in Normal and Neoplastic Breast Development

Annual Report 9/29/1996

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Introduction

I. Nature of the problem

In the past several years, it has been shown that oncogenes contribute to the pathobiology of breast cancer. Mutational activation of the *int-3* oncogene, has been shown to contribute to experimental mammary gland tumorigenesis in mouse. Several human orthologues of the *int-3* gene have been implicated in human cancers. There is strong evidence that the int-3 protein regulates the cell fate decisions required for the morphogenesis and functional differentiation of the mouse mammary gland. Despite this evidence, work on the role of the *int-3* gene in breast cancer is still in its infancy. Extensive studies on other int-3 family members (lin-12/Notch) in organisms more tractable to genetic analysis such as Drosophila and C. elegans, demonstrates the evolutionary conservation of these proteins and their fundamental importance in cell fate decisions.

The proposed research will investigate the role of the int-3 protein in the normal physiology of breast development and study the biochemical properties that are important for int-3 transforming activity. This information will broaden our understanding about the events which control normal mammary gland development and how alterations in those events can lead to neoplastic growth of the mammary gland.

II. Background

Mouse mammary tumor virus induces breast cancer in mouse by insertional mutagenesis. In tumors, viral integration can result in activation of the *int-3* gene by promoter insertion and results in expression of a truncated *int-3* gene product (2.3 kb RNA) (1). The nucleotide sequence of this truncated cDNA revealed homology with the Notch/lin-12 gene family (2). However the full length cDNA of the *int-3* gene has not been cloned. Several lines of evidence confirm a role for int-3 in mammary tumorigenesis. Transfection of a recombinant *int-3* genomic DNA fragment, encoding the truncated oncoprotein, into the HC11 mouse mammary epithelial cell line induces anchorage-independent growth in soft agar (2). Expression of this same genomic fragment *in vivo* as a transgene in a transgenic mouse strain is associated with arrest of normal mammary gland development and impaired differentiation (3), intraductal hyperplasia of mammary epithelium, and a high incidence of focal mammary tumors

(adenocarcinomas) (4). It has also been reported that the normal *int-3* gene is endogenously expressed in the mouse mammary gland (5).

Int-3 is related to the Notch/lin-12 family of proteins. The Notch/lin-12 protein family currently consist of eleven members, Notch (Drosophila) (6), lin-12 and glp-1 (C. Elegans) (7, 8, 9), Xotch (Xenopus) (10), Notch 1, 2, 3 and int-3 (Mouse) (2, 11-15), Notch 1 and 2 (Rat) (16,17), NOTCH 1 and 2 (Human) (18, 19). These genes encode for transmembrane receptor proteins. The extracellular domain of Notch/lin-12 family members contains variable numbers of EGF (epidermal growth factor) like repeats and other cysteine rich repeats named lin-12/Notch repeats(26). The intracellular domain of all Notch/lin-12 family members contains several copies of a repeat sequence, named cdc10 or ankyrin repeat. The cdc10 repeats have recently been implicated as a protein-protein interaction domain. The intracellular domain of this family of proteins also contains a PEST sequence, a nuclear localization signal, and an opa repeat. PEST sequences are found in proteins which are rapidly degraded or may also represent potential phosphorylation sites. An opa repeat is a protein domain that is rich in glutamine and is commonly found in transactivating domains of transcription factors or transcription factor binding proteins (27). The Notch and lin-12 proteins are required for cell-cell interactions that play a pivotal role in cell-fate decisions. For instance, the mechanisms that control how a group of equivalent progenitor cells give rise to a group of cells each with their particular fate. The fundamental importance of these genes during development has been demonstrated by genetic analysis of lin-12, Notch and Xotch mutants (20-24). In the mouse, null mutants of Notch 1 and 2 are lethal during embryonic development, although the exact cause for this premature death is not known (25).

Genetic and molecular analysis have identified several proteins that participate in Notch signaling. Drosophila *Delta* (28) and *Serrate* (29) and C. elegans *Lag-2* (30)and *Apx-1* encode a family of structurally related ligands for the Drosophila Notch and C.elegans lin-12 and glp-1 receptors respectively. These ligands are transmembrane proteins, containing EGF-like domains and a cysteine rich DSL (Delta-Serrate-Lag-2) domain within the extracellular part of the protein. Recently, mouse homologues of these ligands have been cloned, *Jagged-1* (31) and *Dll-1* (32). These ligands have been demonstrated to regulate Notch receptor activity through cell-cell interactions. The products of three Drosophila genes, deltex, disheveled and suppressor of hairless (Su(H))have been shown to interact with the intracellular domain of Notch and may thus participate in the intracellular signaling pathway of Notch (33,34) Furthermore, genetic analysis has revealed similar phenotypes in certain Deltex and Notch mutants.

Deletion of the extracellular part of Notch, Xotch and lin-12 proteins results in a dominant gain of function mutation (20-22). The truncated gene product encoding for the intracellular part of the receptor exhibits constitutively activated protein function. The phenotype observed in this class of mutants suggests that the truncated gene products delay cell determination and thereby increase the proportion of uncommitted stem cells, leading to a prolonged lifetime of the cell or to a greater number of descendants (20-22). By analogy to the function of other Notch/lin-12 family members in lower organisms, one can speculate that delay in differentiation and accumulation of pluripotent proliferative stem cells would result in a growth advantage, thereby increasing the probability for secondary oncogenic mutations. This model would propose that Notch proteins contribute to oncogenesis by stimulating stem cell growth and blocking differentiation.

Studies on the Notch protein in Drosophila, demonstrated that the intracellular part of the Notch protein is translocated to the nucleus when a truncated Notch protein (corresponding to the intracellular part of the protein) is expressed as a transgene in Drosophila embryos (20). Based on the hypermorphic effect of the deletion mutants, and on the presence of a nuclear translocation signal in the intracellular domain of the protein, a hypothetical model would be that ligand binding to the receptor would result in cleavage of the intracellular domain of the receptor and subsequent translocation to the nucleus, where it could interact with its substrate.

Notch/lin-12 gene family members have been implicated in human tumorigenesis. Alteration of NOTCH-1 (also named TAN-1) has been associated with a T lymphoblastic neoplasm (18). The mutation of the NOTCH-1 gene in T lymphoblastic lymphomas is caused by a translocation that results in expression of a truncated gene product. TAN-1 mutations are analogous to the *int-3* activating mutations as a result of MMTV insertion, as well as to the dominant gain of function mutations of Notch, lin-12 and Xotch. Furthermore, human NOTCH-1 and NOTCH-2 (also named hN) were found to be overexpressed in human cervical carcinomas (19).

III. Purpose

The overall goal of the work proposed here is to understand in molecular details of the function of the int-3 protein in mammary epithelial cells and during mouse mammary gland development, with the *long term goal* of understanding the role of the *int-3* gene in mammary tumorigenesis.

Body

I. Technical Objectives

The overall goal of the work proposed here is to understand in molecular details of the function of the int-3 protein in mammary epithelial cells and during mouse mammary gland development.

We proposed three specific aims to investigate the mechanisms of the functions of int-3 protein in a relatively simple and biological context:

1. Compare the normal int-3 protein to activated int-3 oncoproteins

We intend to first determine the primary sequence of the normal int-3 protein and evaluate whether the normal *int-3* gene encodes a Notch-like transmembrane receptor. We will then compare the biochemical and physiological properties of the normal int-3 protein with that of truncated int-3 oncoproteins. Our aim is to determine what structural alterations lead to oncogenic activation of int-3 and to evaluate whether activated int-3 proteins act at the cell surface and/or the nucleus.

2. Identifying proteins that interact with the intracellular domain of int-3.

To elucidate the molecular mechanisms of the oncogenic effect of activated int-3, we will identify proteins or protein complexes that interact with int-3. Three approaches will be used: (1) affinity purification of associated proteins, (2) library screening with radiolabled int-3 proteins, and (3) a yeast two-hybrid genetic screen for int-3 associated proteins.

3. Analysis of int-3 expression pattern in the murine mammary gland.

The fact that overexpression of activated int-3 in mouse mammary gland has profound effects on the development of the gland suggests a role of nomrla *int-3* gene products in mammary gland development. We will determine whether *int-3* gene is normally expressed in the mamamry gland. Molecular and immunohistochemical techniques will be used to evaluate where and when in the normal mammary gland the *int-3* gene is expressed.

To fullfill these specific aims, we have proposed five technical objectives for a period of four years.

Task 1: Characterize the full length *int-3* cDNA. (Months 1-12)

Task 2: Study the production, processing and subcellular localization of normal int-3 proteins and int-3 oncoprotein in mammary epithelial cells. (Months 3-18)

Task 3: Evaluate the transforming potential of int-3 proteins. (Month 9-18)

Task 4: Identify proteins that interact with the intracellular domain of int-3. (Month 12-48)

Task 5: Analysis of the int-3 expression pattern in the murine mammary gland. (Months 12-48)

II. Experimental Results

This annual report describes the progress we have made during the first 24 months of this grant. The progress reported here will be related to the original tasks set out in the Statement of Work.

1. Characterize the full length *int-3* cDNA. (Months 1-12)

The *int-3* oncogene has been classified in the lin-12/Notch protein family solely on the basis of its homology to the intracellular part of the lin-12/Notch family members. There has been no direct evidence that demonstrates that the full length *int-3* encodes for a transmembrane protein. By cloning the full length *int-3* cDNA we have demonstrated that the *int-3* gene encodes for a transmembrane protein, homologous to the lin-12/Notch family of transmembrane proteins. We have proposed to name the full length gene *Notch4*, and reserve the *int-3* nomenclature when referring to the truncated and oncogenic form of the gene.

A. Cloning truncated *int-3.* We have cloned the truncated *int-3* gene that encodes for the intracellular part of the protein and corresponds to the int-3 mammary oncogene (see paper, Appendix A)

B. Cloning of the full length int-3 We have used the PCR based method of RACE (Rapid Amplification of cDNA Ends) (35,36) to clone sequences that are located 5' from the truncated *int-3* (the truncated *int-3* transcript is localized at the 3' end of the gene). To clone the *int-3* full length cDNA, we have screened a mouse lung cDNA library. This choice of cDNA library was based on our findings of the mRNA expression analysis of *int-3* . Initially, the probes that were used in this screening analysis were derived from the cloned truncated *int-3*, as well as from the clones obtained by RACE. Positive clones were purified and sequenced and used as probes in successive rounds of screening in order to obtain the full length cDNA of the *int-3* gene. The length of the *int-3* mRNA is approximately 6.7kb based on our Northern blot analysis. The *int-3* sequence has been analyzed for its homology with other lin-12/Notch family members, and has a high overall homology to the other known mouse *Notch* 1, 2 and 3 genes. The predicted amino acid sequence encodes for a transmembrane protein with a intracellular domain containing six ankyrin repeats, a transmembrane domain, and a extracellular domain containing three Notch/lin-12 repeats and twenty nine EGF-like repeats (see paper, Appendix A).

C. Sequencing *int-3* cDNAs We have sequenced the full length gene of int-3. According to its DNA and amino acid sequences, the full length gene of int-3 is a bona fide Notch/lin-12 family gene. It bears all the typical tructural motifs found in Notch/lin-12 family genes.

2. Study the production, processing and subcellular localization of normal int-3 proteins and int-3 oncoprotein in mammary epithelial cells. (Months 3-18)

The *int-3* gene was discovered in mouse mammary tumors, induced by MMTV infection. Insertional mutagenesis by MMTV results in expression of a truncated int-3 protein product that is able to transform mammary epithelium cells both *in vivo* and *in vitro*.

A. Epitope-tagging of int-3 proteins. Since antibodies were not immediately available against the int-3 protein (aim 3), we have added an epitope to the coding region of truncated int-3(37). The epitope we have chosen to add to the int-3 protein is derived from the influenza HemAgglutinin (HA) protein and is recognized by a monoclonal antibody 12CA5 (38). *Int-3* cDNA was cloned into phagemid vectors that contain the sequence encoding the HA epitope situated downstream. Single strands were generated from the phagemid and used in a site directed mutagenesis protocol with an oligonucleotide designed to loop-out the sequence between the last *int-3* codon and the first HA codon, to create in frame a cDNA encoding the int-3/HA fusion protein. The HA epitope was fused to the carboxy terminus of the int-3 protein. This method allowed us to detect ectopically expressed int-3/HA fusion protein in transfection experiments, using anti HA antibodies in immunoblot analysis (Appendix B). The molecular weight of epitope tagged int-3 is approximately 60 KD.

B. Generation of int-3 expressing cell lines. An *int-3/HA* fusion construct was sub-cloned into a murine leukemia virus (MLV) based vectors, denoted LNCX vectors (39). These vectors utilize the cytomegalovirus immediate early promoter/enhancer to drive expression of the *int-3* gene and the retroviral LTR to drive expression of the *neo* gene, which confers resistance to the drug G418. We have also generated several control retroviral vectors, such as vectors not containing *int-3*, vectors containing an unrelated HA tagged protein, and vectors containing *int-3* not HA tagged. This last construct allowed us to determine that the HA tagging does not interfere with the transforming activity of int-3 (see below). In order to evaluate the transforming potential of int-3, a mammary epithelial cell line programmed to express int-3 was generated by infection with retroviral expression vectors. High-titer helper free retroviral stocks were generated using the BOSC23 ecotropic virus packaging cell line (40), media was collected and used to infect the mammary epithelial cell lines TAC-2 (43). The int-3 stably infected TAC-2 mammary epithelial cell lines express copious amounts of recombinant int-3 proteins as was determined by immunoblot analysis (Appendix C). The expression level of the int-3 recombinant proteins in TAC-2 cells can be increased by treatment of the cells with Sodium Butyrate (Appendix D). In addition, four int-3 deletion mutants were generated. The deletion mutants have either the amino terminal end (-NT), the cdc-10 repeats (-cdc), the carboxy terminal end (-CT), or the amino and carboxy terminal end (-

NT-CT) of the *int-3* gene deleted. All four int-3 deletion mutants were epitope tagged at the carboxy terminus. TAC-2 cell lines were generated that express the different int-3 deletion mutants as was determined by immunoblotanalysis (Appendix D).

C. Biochemical analysis of int-3(Notch4) proteins produced in cell lines. The deduced amino acid sequence of the full length Notch4 protein predicts that Notch4 is a putative transmembrane protein. We will investigate this hypothesis by studying the intracellular localization as well as the glycosylation state of the Notch4 protein. These studies may confirm the predicted transmembrane nature of the Notch4 protein. The biochemical properties of Notch4 will be investigated once mammary epithelial cell lines have been generated that are programmed to express Notch4. A full length Notch4 transcript was assembled and the Notch4 protein was detected in protein lysates of transiently transfected 293T cells (Appendix B). The molecular weight of epitope tagged Notch4 is approximately 215 KD. Immunofluorescence experiments on 293T cells transiently transfected with epitope tagged Notch4, cells demonstrated plasma membrane staining (data not shown).

D. Subcellular localization of int-3 proteins The intracellular localization of truncated int-3 was investigated in transiently transfected cells (293T and HeLa cells). We have investigated the intracellular localization by indirect immunofluorescence (45) using the anti-HA antibodies, and have found nuclear localization of truncated int-3 (as reported in annual report 9/95). Several deletion mutants have been generated (see above and Appendix D) in order to determine which part of the truncated int-3 protein is responsible for its nuclear translocation. These initial studies pointed out two domains that may contain a nuclear translocation signal, one N-termnal and one C-termnal of the ankyrin repeats. Interestingly, deletion of the ankyrin repeats alone did not affect the nuclear localization. Preliminary data suggest that a protein consisting solely of the int-3 ankyrin repeats resides in the cytoplasm. These studies will also allow us to investigate whether transformation coalesces with cytoplasmic or nuclear localization of the int-3 protein.

3. Evaluate the transforming potential of int-3 proteins. (Month 9-18)

A. Generate cell lines expressing int-3 proteins We have generated TAC-2 cell lines that stably express int-3 proteins.

B. Evaluate the transformed properties of int-3 expressing cell lines We have proposed to study mammary transformation by int-3, we have, instead established a biological assay for int-3 based on its ability to block ductal morphogenesis of cultured mammary epithelial cells. The biological activity of int-3 was studied in the TAC-2 mouse mammary epithelial cell line. TAC-2 cells were isolated based on their ability to undergo branching morphogenesis when grown in a collagen gel matrix (46). This differentiation of the TAC-2 cells into tree-like structures can be

specifically induced by treatment with Hepatocyte growth factor (HGF) (46). TAC-2 cells that express int-3 protein fail to undergo branching morphogenesis, when grown in the presence of HGF (Appendix E). Control cell lines, TAC-2 cells expressing Lac-Z, and TAC-2 cells expressing the mammary oncogene Wnt1, were still able to differentiate into tree-like structures when induced with HGF (data not shown and Appendix E), suggesting that the int-3 induced block of differentiation is specific. Since int-3 is able to block branching morphogenesis of TAC-2 cells, we investigated the growth characteristics of the different TAC-2 cell lines and found no differences in growth rate (day 2) or post-confluence growth rate (day 6) between int-3 expressing TAC-2 cells and control TAC-2 cells or TAC-2 cells expressing Wnt1, in the presence or absence of HGF (Appendix F). The growth of TAC-2 cells was measured by a commercially available biochemical assay. These experiments suggest that the int-3 oncoprotein prevents differentiation of mammary epithelial cells without affecting the growth characteristics of these cells. The above described preliminary data are reminiscent of the mammary gland phenotype of the *int-3* transgenic mice. These animals that overexpress the *int-3* oncogene in the mammary gland fail to develop a normal mammary gland (no branching morphogenesis) and the mammary epithelial cells present display an undifferentiated phenotype.

4. Identify proteins that interact with the intracellular domain of int-3. (Month 12-48)

A. Identify int-3 binding proteins using yeast two-hybrid genetic screen (corresponds to task 4d) To study the downstream signaling pathway of Notch4 receptor, we planned to carry out yeast two hybrid screen to search for proteins that interact with the intracellular domain of Notch4. We first generated two bait constructs for two hybrid screen. They contained either the whole intracellular domain or the ankyrin repeats region fused to LexA DNA binding domain. Unfortunately, when we tested the two constructs for the ability to activate LacZ reporter gene expression in yeast on their own, the ankyrin repeat construct gave very robust positive signal while the other construct produced a medium level positive signal. We tried to tackle this problem with bait constructs containing a series of deletion mutants of the intracellular domain of int-3. We were hoping to get rid of the transcriptional activity of the bait yet keep the intracellular domain as much as possible so that we could still identify Notch4 binding proteins through two hybrid screen. However, all the deletion mutant constructs we have tested are more or less active in β-galactosidase assay (data not shown). We are currently trying different approaches to get around this technical difficulty. These approaches include testing our bait contructs in different yeast strains, testing the bait constructs for HIS3 reporter gene activation and adding 3-AT in yeast culture medium to suppress the leakiness of the HIS3 gene. Once an appropriate bait construct is generated, it will be used to screen a mouse embryonic cDNA library.

5 Analysis of the int-3 expression pattern in the murine mammary gland. (Months 12-48)

We will analyze the expression pattern of the Notch4 protein as well as the *Notch4* mRNA. Such analysis will give us further insights in the function of the Notch4 protein. We will study the expression pattern of *Notch4* in different mouse tissues to determine if the Notch4 protein is tissue specifically expressed, and we will investigate whether *Notch4* is expressed in the mammary gland and whether this expression is developmentally regulated.

A. Generate int-3 specific antibodies To study the biological and biochemical behavior of the Notch4 and int-3 proteins, we have successfully generated rabbit antiserum against a GST fusion protein of Notch4. An intracellular fragment of Notch4 protein, which is C-terminal to the ankyrin repeats, was fused to GST protein. This fragment is the least homologous domain among different murine Notch proteins and therefore, it was chosen as immunogen to avoid cross-reactivity with other proteins. The specificity of the antiserum as well as its ability to recognize int-3/Notch4 was analyzed in immunoblot analysis. Ectopically expressed int-3 and Notch4 proteins, were used to test specificity of the rabbit antiserum (Appendix B). The appropriate total immune serum titer for detection in immunoblot experiments has been determined (Appendix B). Affinity purified immune serum has been generated using a GST-int-3 column, and titers of affinity purified immune serum have been determined. We have also tested the antiserum for its ability to immunoprecipitate int-3 and Notch4 proteins. Cells overexpressing int-3 and Notch4 proteins were first metabolically labelled with ^{35}S, and then lysed to prepare cell extracts that were used for immunoprecipitation. int-3 protein has been successfully pulled donw by our antiserum (Appendix G). We are currently trying to immunoprecipitate the full length Notch4 protein.

B. Northern blot analysis of int-3 expressed in mammary tissue (corresponds to task 5c). We have not analyzed mammary gland expression but have determined the expression pattern of int-3/Notch4 in adult and embryonic tissues. *Notch4* mRNA expression was studied by Northern blot analysis and in situ hybridization using probes derived from the 3' UTR (Appendix A). The *Notch4* gene encodes for a 6.5 kb transcript that is highly expressed in lung, heart and kidney in adult tissues. Several shorter *int-3* transcripts were observed in adult testis, and are the products of aberrant transcriptional events in post-meiotic spermatids (Appendix A). The *Notch4* transcript is expressed at all stages of mouse development (day 6.5 to 15.5). In situ hybridization (using the same probe as in the Northern blot analysis) was performed to determine the cellular origin of *Notch4* expression during mouse development, and revealed endothelial specific expression. In situ hybridization on adult lung tissue was performed and revealed endothelial cell specific expression of *Notch4*.

Conclusions

The data presented in this annual report represent our progress in the experiments outlined in the specific aims of the research proposal. As outlined in the statement of work in the research proposal, we have largely completed the aims as scheduled for months 1-24.

The cloning of *int-3/Notch4* is completed. The deduced amino acid sequence of the Notch4 protein displays high homology with the *Notch/lin-12* gene family members, identifying this gene as the fourth murine *Notch* gene. We propose to rename this gene *Notch4*, reserving the *int-3* nomenclature for the activated and truncated form. The deduced amino acid sequence of Notch4 reveals 29 EGF-like repeats and 3 Notch/lin-12 repeats in the extracellular domain, a transmembrane domain, and an intracellular domain containing six ankyrin repeats. The above data confirms the initial hypothesis that *int-3* is a bonafide member of the *Notch/lin-12* gene family.

The truncated *int-3* gene and the full length *Notch4* gene has been assembled, epitope tagged, and cloned into an eukaryotic expression vector. Cell lines have been generated that are programmed to express the truncated int-3 protein. The truncated int-3 protein expressed in mammary epithelial cells was detected by Western blot and Immunofluorescence analysis. A biological assay was identified that can evaluate specifically for int-3 activity. In this assay, the int-3 oncoprotein can prevent growth factor mediated differentiation, in an analogous matter to the *int-3* transgenic mouse phenotype. This tissue culture model will be used to study in detail the biochemical characteristics of int-3

The expression analysis of the *Notch4 mRNA* was analyzed in adult tissues as well as during mouse development. The *Notch4* transcript consists of a 6.5 kb mRNA species, that is specifically expressed in embryonic and adult endothelial cells.

A rabbit polyclonal antibody was raised successfully against the intracellular domain of Notch4, and the specificity and immune titer was determined.

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Appendices

Appendix A We have included a published manuscript which describes our progress:
Uyttendaele, H., G. Marrazzi, G. Wu, Q. Yan, D. Sassoon, and J. Kitajewski. 1996. *Notch4/int-3*, a mammary proto-oncogene, is an endothelial cell-specific *Notch* gene. **Development** 122: 2251-2259.

Appendix B Immunoblot analysis of epitope tagged int-3 and Notch4 proteins

Appendix C Immunoblot analysis of TAC-2 cells programmed to express int-3

Appendix D Immunoblot analysis of TAC-2 cells programmed to express int-3 deletion mutants

Appendix E TAC-2 branching morphogenesis assay

Appendix F TAC-2 growth assay

Appendix G Immunoprecipitation of int-3 and int-3HA proteins

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Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian *Notch* gene

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SUMMARY

The *int-3* oncogene was identified as a frequent target in Mouse Mammary Tumor Virus (MMTV)-induced mammary carcinomas and encodes the intracellular domain of a novel mouse *Notch* gene. To investigate the role of the *int-3* proto-oncogene in mouse development and carcinogenesis, we isolated cDNA clones corresponding to the entire coding potential of the *int-3* proto-oncogene. We propose to name this gene *Notch4* and reserve the *int-3* nomenclature for references to the oncogenic form. The deduced amino acid sequence of Notch4 contains conserved motifs found in Notch proteins; however Notch4 has fewer epidermal growth factor (EGF)-like repeats and a shorter intracellular domain than other mouse Notch homologues. Comparison of the coding potential of the *int-3* gene to that of *Notch4* suggests that loss of the extracellular domain of Notch4 leads to constitutive activation of this murine Notch protein. In situ hybridization revealed that *Notch4* transcripts are primarily restricted to endothelial cells in embryonic and adult life. Truncated *Notch4* transcripts were detected in post-meiotic male germ cells. The distinct Notch4 protein features and its restricted expression pattern suggests a specific role for *Notch4* during development of vertebrate endothelium.

Key words: Notch, int-3, endothelial cells, mammary oncogene

INTRODUCTION

The *int-3* gene was originally identified on the basis of its oncogenic effects in the mouse mammary gland. *int-3* is a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors (Gallahan and Callahan, 1987; Robbins et al., 1992; Sarkar et al., 1994). Tumor-specific transcripts derived from the *int-3* gene encode a protein homologous to the intracellular part of the Notch family of cell surface receptors. Exogenous expression of the int-3 oncoprotein has been shown to affect the growth and development of mammary epithelial cells. Overexpression of the int-3 oncoprotein in mouse mammary epithelial cells (HC11) promotes anchorage-independent growth (Robbins et al., 1992). Expression of *int-3* as an MMTV-LTR-driven transgene in the mouse mammary gland results in abnormal development of the mammary gland and rapid development of undifferentiated mammary carcinomas (Jhappan et al., 1992). In the normal mouse mammary gland, endogenous int-3 protein has been detected in mammary stroma and epithelium (Smith et al., 1995).

Members of the *Notch/lin-12* gene family were first identified in *Drosophila* and *Caenorhabditis elegans* through genetic analysis of mutations that alter cell fate decisions (for reviews see Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas and Simpson, 1991; Greenwald and Rubin, 1992). *Drosophila Notch* regulates multiple cell fate decisions that involve cell-cell interactions during fly development, for instance, control of cell fate decisions involving neural/epidermal specification in proneural clusters (Artavanis-Tsakonas and Simpson, 1991). The *C. elegans* lin-12 and glp-1 proteins are structurally related to Notch and are also involved in cell fate specifications during development in the nematode (Greenwald, 1985; Yochem and Greenwald, 1989). Genetic analysis of *Notch/lin-12* genes suggests that this family of genes controls binary cell fate decisions and inductive signaling that depend on cell-cell interactions (reviewed by Artavanis-Tsakonas et al., 1995; Greenwald, 1994; Greenwald and Rubin, 1992). Alternatively, *Notch/lin-12* genes have been proposed to block cell differentiation, thus maintaining the competence of cells for subsequent cell-fate determination (Coffman et al., 1993; Fortini et al., 1993).

Notch/lin-12 genes encode transmembrane receptor proteins characterized by highly repeated, conserved domains. The amino terminus of Notch proteins encodes the extracellular domain and contains as many as 36 repeats of an EGF-like motif involved in ligand binding (Rebay et al., 1993) and three tandem copies of a Notch/lin-12 sequence motif of unknown function. The intracellular portion of Notch proteins is characterized by six tandem copies of a cdc10/ankyrin motif, thought

to be a protein-protein interaction domain (Michaely and Bennett, 1992) and a PEST sequence motif which may represent a protein degradation signal (Rogers et al., 1986). In several systems, truncated forms of Notch/lin-12 proteins that contain an intact intracellular domain without most of the extracellular domain behave as constitutively activated receptors (reviewed by Artavanis-Tsakonas et al., 1995; Greenwald, 1994). The human Notch 1 orthologue, TAN-1, was first identified in independently isolated translocation breakpoints in acute T lymphoblastic leukemia, and is predicted to encode a truncated product that has an intact intracellular domain but lacks most of the extracellular domain (Ellisen et al., 1991). Similarly, the int-3 oncoprotein encodes the intracellular domain of a Notch-like protein and thus has been proposed to act as an activated Notch receptor (Robbins et al., 1992).

Based on sequence similarity to *Drosophila Notch*, additional *Notch*-related genes have been isolated from mammals, including mouse (Franco Del Amo et al., 1993; Lardelli et al., 1994; Lardelli and Lendahl, 1993; Reaume et al., 1992), rat (Weinmaster et al., 1992; Weinmaster et al., 1991) and human (Ellisen et al., 1991; Stifani et al., 1992; Sugaya et al., 1994). To date, three *Notch* homologues, *Notch1*, *Notch2* and *Notch3*, have been identified in the mouse, and their embryonic expression patterns display partially overlapping but distinct patterns of expression that are consistent with a potential role in the formation of the mesoderm, somites and nervous system (Williams et al., 1995). Abundant expression of *Notch1*, *Notch2* and *Notch3* is found in proliferating neuroepithelium during central nervous system development. Targeted disruption of the *Notch1* gene in mice results in embryonic death during the second half of gestation (Conlon et al., 1995; Swiatek et al., 1994) and homozygous mutant embryos display delayed somitogenesis as well as widespread cell death, preferentially in neuroepithelium and neurogenic neural crest (Conlon et al., 1995; Swiatek et al., 1994).

The gene products of *Drosophila Delta* (Vassin et al., 1987) and *Serrate* (Fleming et al., 1990) and *C. elegans Lag-2* (Henderson et al., 1994; Tax et al., 1994) and *Apx-1* (Mello et al., 1994) are thought to act as ligands for Notch proteins. In the mouse, the orthologue of *Delta*, referred to a *Dll1* (*Delta-like gene 1*), is expressed during embryonic development in the paraxial mesoderm and nervous system in a pattern similar to that of mouse *Notch1* (Bettenhausen et al., 1995). A murine *Serrate*-related gene named *Jagged* has been identified and is partially co-expressed with murine *Notch* genes in the developing spinal cord (Lindsell et al., 1995).

We report here the identification and expression analysis of a fourth murine *Notch* homologue, which we propose to name *Notch4*, reserving the *int-3* nomenclature for the truncated oncogene. Although the intracellular domain of the int-3 oncoprotein shares homology with the Notch/Lin-12 protein family, we now provide a comparison of the full-length Notch4 protein with that of the int-3 oncoprotein. The activated int-3 protein contains only the transmembrane and intracellular domain of the Notch4 protein. The predicted amino acid sequence of Notch4 includes the conserved features of all Notch proteins, but Notch4 has seven fewer EGF-like repeats compared to Notch1 and Notch2 and contains a significantly shorter intracellular domain. Notch4 is expressed primarily in embryonic endothelium and in adult endothelium and male germ cells.

MATERIALS AND METHODS

Isolation and sequencing of *Notch4* cDNA clones

A 1680 bp fragment was amplified by PCR from adult mouse testis cDNA (RT-PCR) using specific primers (5′ primer: CGTCCTGCTGCGCTTCCTTGCA and 3′ primer: CCGGTGCCTAGTTCAGATTTCTTA) designed from the *int-3* cDNA sequence (Robbins et al., 1992). This cDNA fragment corresponds to the previously cloned *int-3* oncogene. Two consecutive 5′ RACE reactions (5′-Amplifinder RACE kit, Clonetech) using testis and lung cDNA were done to obtain cDNA clones located 5′ of the *int-3* oncogene. The above described cDNAs were cloned into Bluescript KS (Stratagene) and the TA cloning vector (Invitrogen) and used to generate probes to screen a lung cDNA library (Clonetech). Briefly, nitrocellulose membranes (Schleicher&Schuell) were hybridized in a solution containing 50% formamide, 3× SSC, 100 mM Tris-HCl (pH 7.4), 5× Denhardt's solution, 0.2% SDS and 0.1 mg/ml salmon sperm DNA at 42°C for 14 hours. Filters were then washed in 1× SSC and 0.5% SDS at room temperature followed by washes at 65°C. Positive clones were purified and sequenced to confirm overlapping regions. Novel 5′ restriction fragments of these newly isolated clones were used in consecutive screens in order to obtain the full-length *Notch4* cDNA. All the above described clones were sequenced using the dideoxy termination method (Sanger) with an automatic DNA sequencer (Applied Biosystems). Sequence data from both strands were obtained for the entire *Notch4* cDNA and were analyzed and assembled using computer software (MacVector, Assemblylign).

Northern blot analysis

Total RNA was isolated from adult CD-1 mouse tissues and northern blot hybridization analysis was performed. 20 μg of total RNA was electrophoresed on a 1% agarose gel containing 6% formaldehyde. After electrophoresis RNAs were transferred to a nylon membrane (Duralon-UV membranes, Stratagene) by capillary blotting. ^{32}P-labeled riboprobes were transcribed (Maxiscript in vitro transcription kit, Ambion) from *Notch4* cDNA clones encoding the 5′ or 3′ UTR (untranslated region) or ORF (open reading frame). The 3′ UTR *Notch4* cDNA clone was isolated by RT-PCR and a 440 bp restriction fragment of this cDNA was used as riboprobe. Hybridization solution contained 60% formamide, 5× SSC, 5× Denhardt's solution, 1% SDS, 20 mM NaH_2PO_4 (pH 6.8), 0.1 mg/ml salmon sperm DNA, 100 μg/ml yeast tRNA, 10 μg/ml poly(A) mRNA and 7% dextran sulfate and was done for 14 hours at 65°C. Washing solution contained 2× SSC and 1% SDS and was done at room temperature and 50°C for 15 minutes each, followed by a 2 hour wash at 80°C with a solution containing 0.2× SSC and 1% SDS. Membranes were exposed to X-ray film (X-OMAT AR, Kodak). The integrity of the RNA, as well as comparable amounts of RNA, were tested by rehybridization with a GAPDH probe.

In situ hybridization

Staged embryos ranging from 9 days post-coitum (d.p.c.) to birth were obtained from timed breedings of CD-1 mice. The morning when the the vaginal plugs appeared was counted as 0.5 d.p.c. Lungs was obtained from adult CD-1 mice. Preparation of tissue and subsequent procedures for in situ hybridization were done as previously described (Marazzi and Buckley, 1993; Sassoon and Rosenthal, 1993). After hybridization, sections were dehydrated rapidly and processed for standard autoradiography using NTB-2 Kodak emulsion and exposed for 2 weeks at 4°C. Analyses were carried out using both light- and dark-field optics on a Leica DA microscope. To avoid potential cross-hybridization with homologous RNAs, we used an antisense ^{35}S-labeled RNA probe corresponding to the 3′ UTR of *Notch4*. Probes were used at a final concentration of 9×10^4 dpm/ml.

RESULTS

Isolation and analysis of *Notch4* cDNA clones

The *int-3* mammary oncogene encodes a truncated protein that is highly homologous to the intracellular part of the Notch receptor proteins. The full-length *int-3* gene, which we will refer to as *Notch4*, had been proposed to encode a novel member of the Notch protein family (Robbins et al., 1992). To prove this hypothesis, we have cloned cDNAs containing the complete coding potential of the *Notch4* gene. Using primers derived from the published sequence of the *int-3* oncogene, RT-PCR was used to isolate a 2.4 kb *int-3* cDNA encoding the putative intracellular portion of the receptor. To obtain cDNA clones encompassing the full coding potential of the normal *int-3* gene, cDNAs were isolated by 5′ RACE and by screening a mouse lung cDNA library. A total of 37 overlapping cDNA clones were analyzed and sequenced to obtain a 6677 bp cDNA sequence. This sequence encodes one long open reading frame of 1964 amino acids, starting with an initiator methionine at nucleotide 347 and terminating with a stop codon at nucleotide 6239. The 6677 bp cDNA corresponds in size to that of *Notch4* transcripts detected by northern blot analysis; thus, we believe the cloned cDNA represents the full-length *Notch4* gene.

Several differences (insertions, deletions and single nucleotide changes) were found between the nucleotide sequence of *Notch4* reported here and the previously published *int-3* nucleotide sequence (Robbins et al., 1992). These differences alter the reading frame in several locations within the intracellular domain and may be a result of differences in sequence analysis or, possibly, of mutations found in the tumor-derived *int-3* transcript (Robbins et al., 1992) that are not found in the *Notch4* gene. The nucleotide sequence of mouse *Notch4* has been deposited with GenBank under the Accession number U43691.

Analysis of the deduced *Notch4* amino acid sequence

Analysis of the deduced amino acid sequence of *Notch4* reveals the presence of conserved domains shared by all Notch proteins (see Fig. 1). Notch4 contains EGF-like repeats, Notch/lin-12 repeats, a transmembrane domain, cdc10/ankyrin repeats and a putative PEST domain. The overall homology between Notch4 and other Notch proteins was determined using GCG (Bestfit, gap weight 3.0, length weight 0.1). The Notch4 protein is approximately 60% similar and 43% identical to other vertebrate Notch proteins and 58% similar and 40% identical to *Drosophila* Notch. Lower homologies were found when compared with the *C. elegans* lin-12 and glp-1 proteins (49% similar and 29% identical).

Two hydrophobic regions in the Notch4 protein sequence were identified by hydropathy analysis (Kyte Doolittle algorithm, data not shown). The N-terminal region contains 19 hydrophobic residues that could function as a signal peptide sequence (Fig. 1) and a putative signal peptidase cleavage site

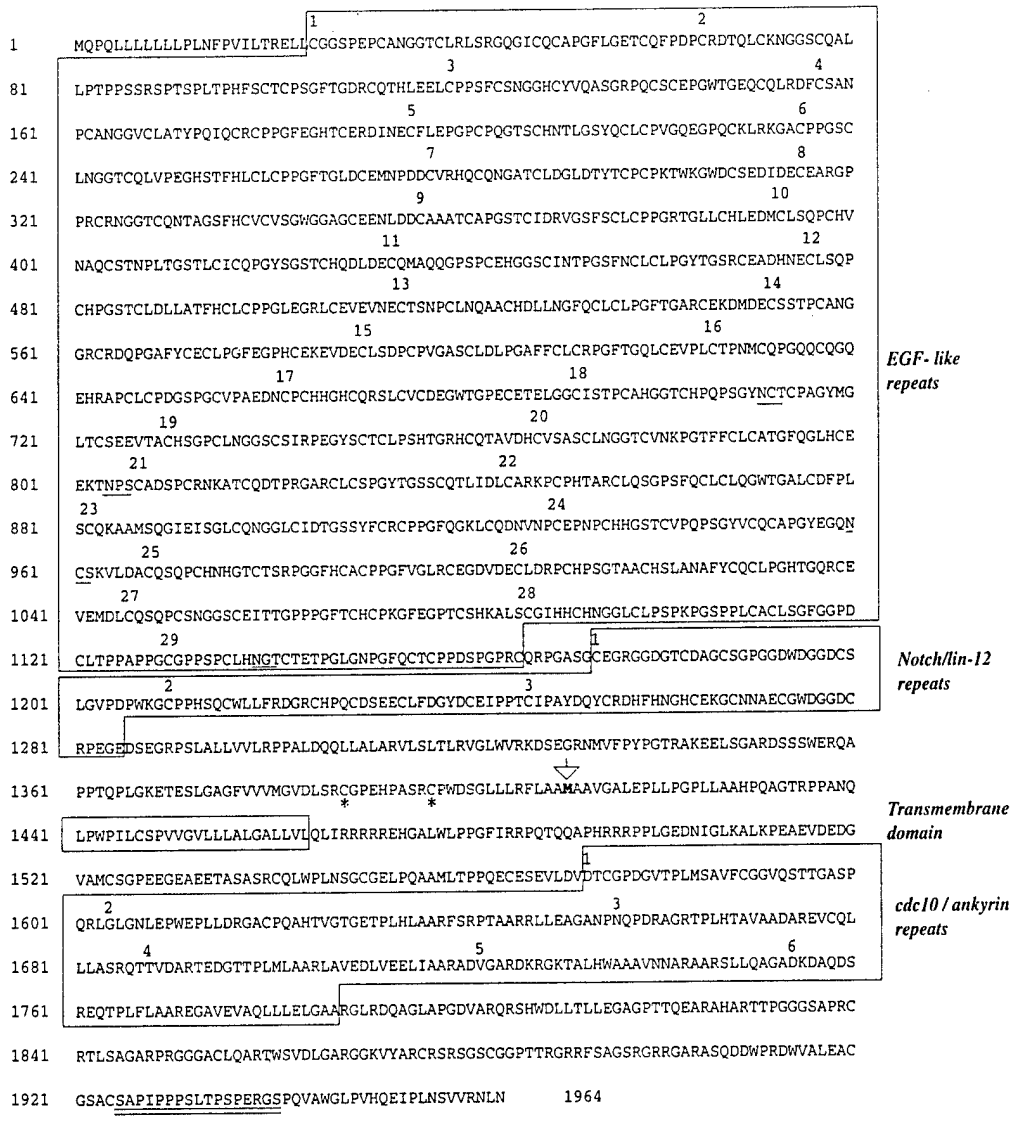

Fig. 1. Deduced amino acid sequence of Notch4 (GenBank accession number U43691). The boxed regions indicate the major structural elements of the Notch family of proteins, as follows: 29 epidermal growth factor(EGF)-like repeats; 3 Notch/lin12 repeats; a transmembrane domain; and 6 cdc10/ankyrin repeats. Putative glycosylation sites are underlined. A putative PEST domain is doubly underlined. The two cysteines thought to promote dimerization are marked with asterisks. The initiating methionine of the int-3 oncoprotein is in bold and marked by an arrow.

was identified at residue 20. A second hydrophobic region from amino acid residues 1441 to 1465 is of sufficient length (25 amino acids) to behave as a membrane-spanning domain and is immediately followed by five consecutive arginine residues that are consistent with a stop transfer signal (Fig. 1).

The extracellular domain of Notch4 contains 29 EGF-like repeats (Figs 1, 2), in contrast to the 36 EGF-like repeats found in murine Notch 1 (Franco Del Amo et al., 1993) and rat Notch 2 (Weinmaster et al., 1992) and to the 34 EGF-like repeats found in murine Notch 3 (Lardelli et al., 1994). EGF-like repeats are defined by a cysteine-rich consensus sequence and generally occur in analogous locations in two different Notch proteins. Since analogous repeats are more homologous to each other than to their neighboring EGF-like repeats, they have been referred to in Notch proteins as equivalent EGF-like repeats. We analyzed the relationship between particular EGF-like repeats of other Notch proteins and those of the Notch4 protein. Fig. 2 schematizes the relationship of EGF-equivalents between Notch4 and Notch1/Notch2. EGF-like repeats 1-13 of Notch4 are equivalent to EGF-like repeats 1-13 of Notch1/Notch2, EGF-like repeats 22-24 of Notch4 correspond to EGF-like repeats 28-30 of Notch1/Notch2 and EGF-like repeats 26-29 of Notch4 are equivalent to EGF-like repeats 33-36 of Notch1/Notch2. Comparison of Notch4 to other Notch proteins revealed no clear-cut identification of the seven particular equivalent EGF-like repeats that are absent in Notch4. The amino acid sequence of equivalent EGF-like repeats has diverged between different Notch homologues and orthologues (Maine et al., 1995), sometimes resulting in loss of a clear-cut equivalent repeat consensus. Six of the unassigned EGF-like repeats of Notch4 appear to be derived from EGF-like repeats 14-27 of Notch1 and Notch2 (Fig. 2). EGF-like repeat 25 of Notch4 may be a hybrid EGF-like repeat derived from parts of EGF-like repeats 31 and 32 of Notch1/Notch2 (Fig. 2). For a discussion of the relationship between Notch3 and Notch1/Notch2 (shown in Fig. 2), see Lardelli et al. (1994).

EGF-like repeats 11 and 12 of *Drosophila* Notch have been shown to be necessary and sufficient for Notch to bind Delta and Serrate proteins in vitro (Rebay et al., 1991). These two equivalent EGF-like repeats are present in Notch4 (Fig. 2). The putative calcium-binding residues (Handford et al., 1991) in EGF-like repeat 11 are also conserved in Notch4 (Fig. 3). The residues between the first and second cysteines of EGF-like repeat 11 have been shown in *Xenopus* Notch to be important in ligand binding and are divergent between Notch proteins (Fig. 3). In this region, Notch4 has additional residues and is unique when compared to other murine Notch proteins. In addition, EGF-like repeats 22-23 of Notch4 have been conserved among murine Notch proteins (EGF-like repeats 28 and 29 of Notch1) and equivalent EGF-like repeats in *Drosophila* Notch are implicated in the regulation of Notch protein function through genetic analysis of the *Abruptex* alleles of Notch (Kelley et al., 1987).

Notch4 also contains three Notch/lin-12 repeats, which are approximately 53% identical to the Notch/lin-12 repeats found in other murine Notch proteins. Between the Notch/lin-12 repeats and the transmembrane domain of Notch4 are two cysteines at positions 1388 and 1397 that are conserved among all Notch proteins and may promote receptor dimerization upon ligand binding (Greenwald and Seydoux, 1990).

The intracellular domain of Notch4 contains the six ankyrin/cdc10 repeats found in other Notch proteins. The ankyrin repeat domain of Notch4 is 48%, 52% and 55% identical to the ankyrin repeat domains of Notch1, Notch2 and Notch3, respectively. In all Notch proteins the number of amino acids between the transmembrane domain and the ankyrin/cdc10 repeats is 110 residues, as it is in Notch4 (Fig. 1). Like other Notch proteins, Notch4 contains a C-terminal PEST domain, albeit of shorter length. In addition, Notch4 lacks a recognizable opa repeat (Fig. 1), such as that found in *Drosophila* Notch. The carboxy-terminal end of Notch proteins, beyond the ankyrin/cdc10 repeats, is the least conserved region among Notch proteins. Within this C-terminal region, Notch4 displays little homology to other Notch proteins and no significant homology to other known proteins. This C terminus is also much shorter in Notch4 (177 residues), than in other Notch proteins (457 residues in Notch1, 437 in Notch2 and 329 in Notch3).

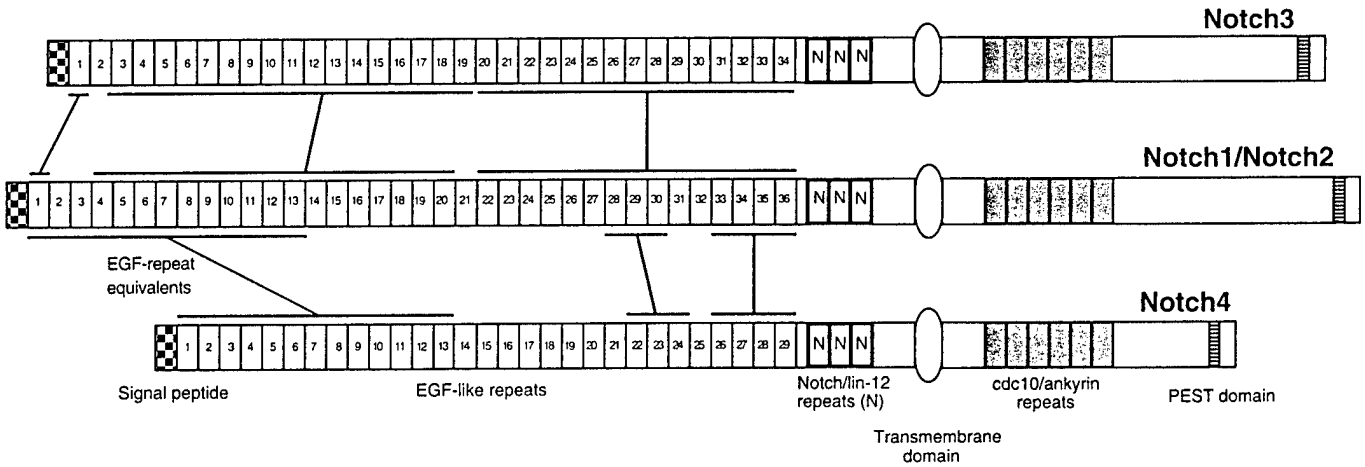

Fig. 2. Schematic structural comparison of the four murine Notch proteins. The EGF-like repeats are numbered according to their position in each different protein. Where equivalent EGF-like repeats can be identified, connecting lines are placed to compare the relationship between these repeats in different Notch proteins (see EGF-repeat equivalents). Notch4 contains seven EGF-like repeats, fewer than Notch1 and Notch2. One of the missing EGF-like repeats (#25) in Notch4 is derived from equivalent repeats #31 and #32 of Notch1/Notch2, creating a novel and hybrid EGF-like repeat. Eight of the EGF-like repeats of Notch4 (#14 to #21) have no identifiable equivalent repeats in Notch1/Notch2. The region of Notch4 from the end of the cdc10/ankyrin repeats to the carboxy terminus is shorter when compared to Notch1, 2 and 3.

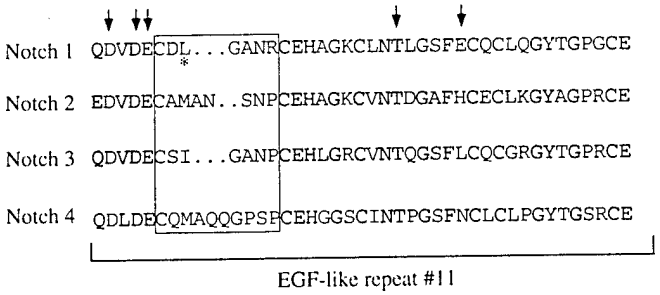

Fig. 3. Amino acid sequence comparison of EGF-like repeat #11 of mouse Notch1, 2, 3 and 4. Residues conserved between the mouse Notch proteins are shaded and the putative calcium-binding sites are marked with arrows. A region within EGF-like repeat #11 of the Notch proteins containing non-conserved and variable numbers of residues is boxed. The leucine to proline mutation in Xenopus Notch that obliterates binding to Delta is marked with an asterisk (*).

Analysis of *Notch4* transcripts in adult tissues

Several adult tissues were examined for the presence of *Notch4* transcripts by northern blot analysis. To minimize cross-hybridization with other mouse *Notch* transcripts, we used a riboprobe derived from the 3′ UTR of *Notch4*. In most tissues analyzed, a single hybridizing species of 6.7 kb was detected (Fig. 4), which roughly corresponds in size to the cloned *Notch4* cDNA. The 6.7 kb transcript is most highly expressed in lung, at lower levels in heart and kidney and at detectable levels in ovary and skeletal muscle. Very low levels of the 6.7 kb transcript were observed in several other adult tissues, including brain, intestine, liver, testis (Fig. 4) and spleen (data not shown). In adult testis, two abundant transcripts of 1.5 kb and 1.1 kb were observed. Thus, *Notch4* expression varies widely in adult tissues. Other than in testis, we did not detect transcript size variation in different tissues.

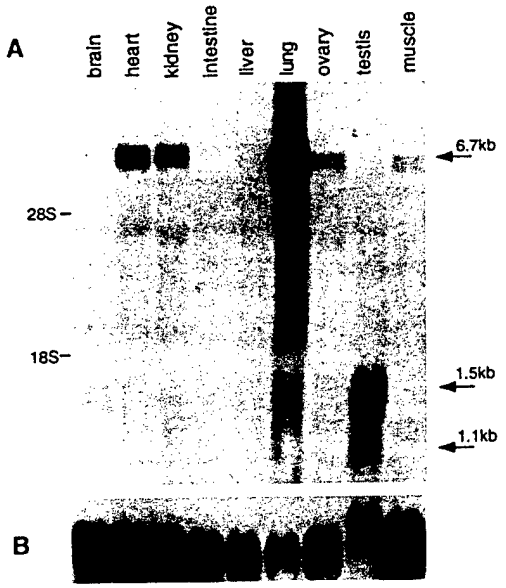

Fig. 4. Expression analysis of *Notch4* in adult mouse tissues. (A) Northern blot using a riboprobe transcribed from the 3′ UTR of *Notch4* (probe D in Fig. 5). (B) The same blot reprobed with a GAPDH probe. The transcript sizes of 6.7 kb, 1.5 kb and 1.1 kb are indicated and were estimated with reference to 28 S and 18 S rRNA migration.

Analysis of testis-specific truncated *Notch4* transcripts

To determine the cell lineage specificity of *Notch4* expression in the murine testis, RNA was analyzed in the germ cell-deficient mouse testis (Fig. 5). Mice that carry two mutations at the white-spotting locus (W/W^v) are devoid of germ cells, but have the normal complement of somatic cell types, including Leydig, Sertoli and peritubular myoid cells (Mintz and Russell, 1957). Heterozygous litter mates ($W/+$) have normal somatic and germ cell complements. Northern blot analysis of total RNA from germ cell-deficient testes (W/W^v) and testes with normal germ cells [$W/+$ and adult (+/+)] was done using a riboprobe derived from the 3′ UTR (probe D in Fig. 5C). Transcripts of 1.5 kb and 1.1 kb were detected in RNA from the testes of adult wild type and $W/+$ mice (Fig. 5A). However, neither transcript was detected in RNA from homozygous mutant testes, suggesting that these transcripts were likely to be specific to the germinal compartment.

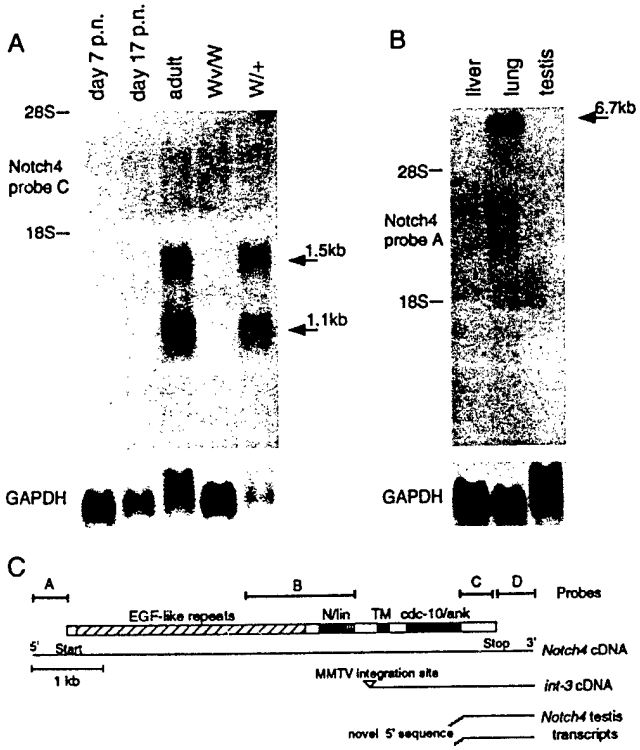

Fig. 5. Expression analysis of *Notch4* testis transcripts. (A) *Notch4* testis transcripts are expressed in post-meiotic germ cells. Northern blot analysis from staged and germ cell-deficient testes with probe C and a GAPDH probe. Note that GAPDH transcripts appear as two isoforms in the adult testis. RNA was isolated from testes of day 7 p.n., day 17 p.n., adult, W^v/W and $W/+$ mice, as indicated. (B) Northern blot analysis of several adult tissues with probe A, derived from the 5′ UTR of Notch4 and a GAPDH probe. (C) Schematic representation of truncated *Notch4* transcripts as compared to the full-length coding potential. Relative positions of probes used in the northern blot analysis are shown. Conserved elements of Notch family proteins are indicated. The MMTV integration site reported by Robbins et al. (1992) is indicated by an arrow. Novel 5′ sequences of testes cDNAs are indicated.

Since spermatogenic differentiation undergoes a characteristic temporal progression, one can use mice testes at specific days of postnatal development to enrich for or eliminate particular germ cell types. Testes from day 7 of postnatal development (day 7 p.n.) mice contain mitotic spermatogonia, while testes from day 17 p.n. mice have entered meiosis and have progressed to spermatocytes (Nebel et al., 1961). Both day 7 p.n. and day 17 p.n. testes lack post-meiotic spermatids. Total RNA from immature and adult testes was analyzed by northern blot hybridization to determine stage-specific expression of *Notch4* transcripts during male germ cell development. Both *Notch4* transcripts of 1.5 kb and 1.1 kb are absent in day 7 p.n. and day 17 p.n. testis, but are present in adult testis (Fig. 5A). These results indicate that the expression of the 1.5 kb and 1.1 kb *Notch4* transcripts is restricted to post-meiotic germ cells.

To determine the nature of the short *Notch4* transcripts in adult mouse testis, northern blot analysis was done using riboprobes derived from different regions of the *Notch4* coding sequence, as well as from 5′ and 3′ UTR (Fig. 5B). A riboprobe derived from the 5′ UTR (probe A in Fig. 5C) failed to hybridize to either the 1.5 kb or the 1.1 kb transcripts (Fig. 5B), whereas this probe did hybridize to the 6.7 kb transcript found in lung RNA (Fig. 5B). However, riboprobes derived from the 3′ UTR (probe D in Fig. 5C) or from cDNA encoding part of the intracellular domain of Notch4 (probe C in Fig. 5C) hybridize to the testis transcripts (Fig. 5A and data not shown). Probes derived from the coding sequence of the extracellular domain of Notch4 (probe B in Fig. 5C) did not hybridize to the testes transcripts (data not shown). To characterize the transcripts expressed in the adult mouse testis, a cDNA library prepared from adult mouse testes RNA was screened using probe C of Fig. 5C. All the clones analyzed encoded the most C-terminal coding sequence and the 3′ untranslated region of Notch4. Two independent clones of distinct size contained novel 5′ sequences unrelated to that found in the full-length *Notch4* cDNA (schematized in Fig. 5C, *Notch4* testis transcripts). Based upon the northern blot analysis described above and the sequence of the cloned testis cDNAs, we believe that *Notch4* transcripts are either derived from an alternate intronic promoter that is active in post-meiotic germ cells or that they may be driven by the same promoter as the 6.7 kb transcript and consist of spliced products derived from a 5′ untranslated region upstream of what we have currently identified. The predicted amino acid sequence of the testis *Notch4* transcripts with the novel 5′ sequence does not contain a methionine that could function as a translation initiator; therefore, these transcripts are unlikely to encode protein products. The testis transcripts may thus represent aberrant transcriptional events in post-meiotic germ cells, as has been described previously (Davies and Willison, 1993).

Expression analysis of *Notch4* during development and in adult lung

A 6.7 kb *Notch4* transcript was detected by northern hybridization in RNA isolated from day 12.5 p.c. mouse embryos (data not shown and Sarkar et al., 1994) and adult lung (Fig. 4). To determine the spatial and temporal pattern of *Notch4* transcript accumulation during development, we examined mouse embryo tissue sections from 9.0 d.p.c. to birth using in situ hybridization. During embryonic development, as well as in postnatal tissues, *Notch4* is highly expressed in endothelial cells. Intense labeling for *Notch4* is observed in embryonic blood vessels at 9.0 d.p.c. (Fig. 6A,B). As shown in Fig. 6C,D, strong labeling is observed over the dorsal aorta, the aortic tract and the pulmonary artery in a 13.5 d.p.c. embryo, while no labeling is detected in the epithelial cells lining the gut (red arrow). At higher magnification, we note that labeling is restricted to the endothelial cells lining the embryonic vessels (Fig. 6D,E) and no labeling is detected in the red blood cells

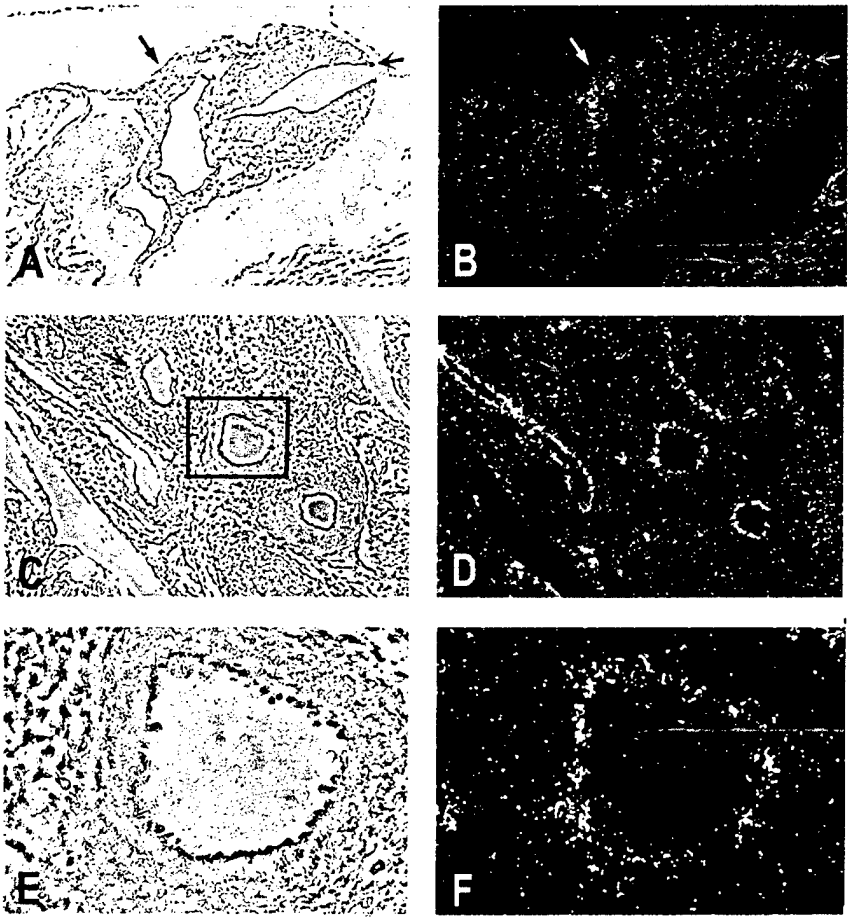

Fig. 6. *Notch4* is expressed in embryonic endothelial cells. (A,B) Phase contrast and dark-field photomicrograph of a horizontal section of a 9 d.p.c. embryo hybridized with a cRNA probe corresponding to *Notch4*. Strong labeling is detectable over the anterior cardinal vein (white/black arrows). Diffuse labeling is also present throughout the developing nervous system and at higher levels over the tip of the neural folds (red arrows). (C-F) Phase and darkfields images of a horizontal section of a 13.5 d.p.c. embryo hybridized for Notch4, showing the venous and arterial system anterior to the lung, including dorsal aorta arch, aortic and pulmonary tract. E and F are higher magnifications of the area framed in C. Embryonic vessels are labeled and, as shown in E and F, labeling is restricted to the endothelial cells lining the vessels. Arrows denote the gut, which does not have a detectable signal in the epithelium.

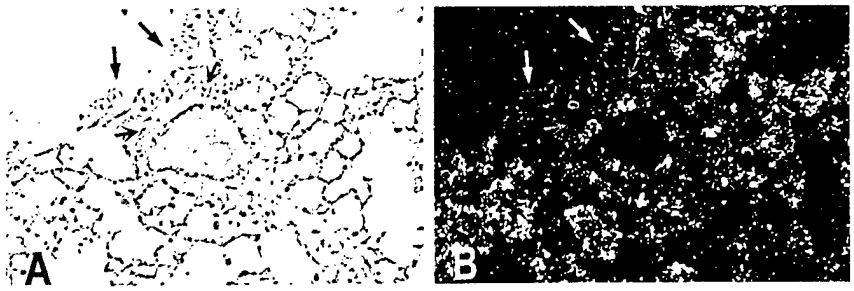

Fig. 7. *Notch4* is expressed in adult lung endothelial cells. (A,B) Phase contrast and dark-field photomicrographs of an adult mouse lung hybridized with a cRNA probe corresponding to *Notch4*. Punctate staining is observed over the alveolar walls, which are predominantly composed of capillaries. No labeling is observed over the pseudostratified squamous epithelium (black and white arrows) nor over the smooth muscle cells (red arrows).

in the vessel. A weak and transient signal is also detectable in the developing nervous system from 9.0 d.p.c. embryos. As shown in Fig. 6A,B, a light diffuse labeling is detected in the developing nervous system and a more distinct signal is observed at the tip of the neural folds. *Notch4* transcripts in the nervous system are still detectable at 11.5 d.p.c., but by 13.5 d.p.c. no labeling for *Notch4* is detectable in the nervous system (data not shown).

Since adult lung exhibited the highest levels of *Notch4* transcripts, in situ hybridization was performed on lung sections to determine whether *Notch4* expression remains endothelial cell-specific in adult life. Intense punctate staining was observed over the alveolar wall, indicative of capillary-specific expression (Fig. 7). The central component of the alveolar wall is the capillary flanked by pneumocyte type I epithelial cells, which line the alveolar lumen (Ross and Reith, 1985). Capillaries are highly localized in the alveolar wall and would give the punctate localized signal observed, as opposed to a more uniform pattern for epithelial cells lining the alveolar cavity. There is clearly no hybridization signal over other cellular components of the lung, that is, pseudostratified squamous epithelium, smooth muscle and connective tissue cells. The endothelial-specific expression probably underlies the abundance of *Notch4* transcripts found by northern blot analysis of highly vascularized adult tissues (lung, heart and kidney in Fig. 4).

DISCUSSION

We report here the identification of a novel mouse gene whose protein product exhibits structural homology with the vertebrate Notch protein family. We have named this gene *Notch4*, as it is the fourth murine *Notch* gene identified. Notch4 contains all the conserved domains characteristic of Notch proteins (Figs 1 and 2). However, Notch4 contains only 29 EGF-like repeats within its extracellular domain as compared to the 36 repeats found in Notch1 and Notch2. In addition, the C-terminal tail of Notch4, beyond the ankyrin/cdc10 repeats, is shorter and unique when compared to all other Notch proteins, but little is known of the function of this region in Notch proteins. Notch4 also contains a distinct EGF-like repeat 11, which has been proposed to be crucial for ligand binding. Structural variation in this repeat and differences in the number of EGF-like repeats between murine Notch proteins, may be important for ligand specificity among the different possible Notch ligands. It must be noted that Notch/lin-12 proteins of varying structure have been demonstrated to be functionally interchangeable; *C. elegans* glp-1 can fully substitute for lin-12 (Fitzgerald et al., 1993) for instance. Therefore, Notch4 may be functionally interchangeable with other murine Notch proteins, despite structural differences between them.

Notch4 is distinct from other Notch family proteins, based on its expression pattern during embryonic development and in the adult mouse. In situ hybridization demonstrates endothelial-specific embryonic expression of *Notch4*. This endothelial-specific expression of *Notch4* remains in the adult mouse. A weak and transient labeling is seen in the neural tube between day 9 p.c. and 11.5 p.c., with a more intense labeling at the tips of neural folds. This region of the neural tube is a highly plastic area where cells will probably participate in the fusion process of the neural tube and/or migrate as neural crest. The *Notch4* expression pattern is in sharp contrast to the expression patterns of *Notch1*, *2* and *3*. These *Notch* genes are expressed in a variety of different embryonic tissues such as the developing brain and spinal cord, presomitic and somitic mesoderm and a variety of epithelial cells and mesenchymal derived tissues (Weinmaster et al., 1991; Williams et al., 1995). *Notch1* is the only other *Notch* gene reported to be expressed in endothelial cells (Reaume et al., 1992; Bettenhausen et al., 1995; Lindsell et al., 1995). Expression of *Notch1* and *4* in endothelial cells might reflect either redundancy of function or distinct biological functions in endothelial development. Endothelial cell-specific expression has recently been reported for a putative Notch ligand, the chick *Serrate* homologue (Myat et al., 1996).

Since Notch proteins have been implicated in binary cell fate specification, regulating how equivalent cells can give rise to cells with different fates, a putative biological function of Notch4 might be to govern the cell fate decisions during endothelial growth and development. In amniotes, endothelial and hematopoietic cells appear synchronously in the blood islands. In zebrafish, lineage data have shown that individual cells of the early blastula can give rise to both endothelial and blood cells, suggesting a common embryonic precursor which has been referred to as the 'hemangioblast'. The occurrence of binary cell fate decision events in the hemangioblast is supported by analysis of the endothelial and/or hematopoietic cell lineages. *Cloche*, *bloodless* and *spadetail* are mutants isolated in zebrafish that display phenotypes defective in either hematopoietic development or both hematopoietic and endothelial development (Stainier et al., 1995). In the mouse, the *Flk-1* and the *Flt-1* genes encode receptor tyrosine kinases that are expressed in embryonic endothelium (Shalaby et al., 1995; Fong et al., 1995). Null mutants for the *Flk-1* gene are defective in endothelial and blood cell development (Shalaby et al., 1995), whereas null mutants for the *Flt-1* gene display only hematopoietic cell development defects (Fong et al.,

1995). Mutational analysis of the *Notch4* gene in whole animals would help to define the role of Notch4 in endothelial cell growth and development.

Alterations in stem cell fate decisions as a result of activated Notch proteins have been proposed to contribute to mitogenic growth of tumor cells. Blocked cell differentiation of fated daughter cells by activated Notch proteins may lead to an increase in the number of cells undergoing cell division, or a prolonged life of the cell. In these cells, the probability of secondary oncogenic mutations that contribute to neoplastic transformation would be enhanced. In the normal mouse mammary gland, endogenous int-3 protein has been detected at low levels in mammary stroma and epithelium (Smith et al., 1995). Although little is known about the nature of stem cells in the mammary epithelium, Notch4 might regulate the fate decisions of mammary epithelial cells. This hypothetical model may explain the phenotype that is observed in *int-3* transgenic mice, which display blocked development of the mammary gland and develop mammary carcinomas at high frequency.

The signal transduction pathways by which Notch proteins function are becoming understood through genetic studies in *Drosophila*. Deltex and Suppresser of Hairless [Su(H)] have been demonstrated to bind to the cdc10 repeats of the intracellular domain of *Drosophila* Notch (Diederich et al., 1994; Fortini and Artavanis-Tsakonas, 1994; Matsuno et al., 1995). More recently the mammalian Su(H) orthologue RBP-Jk, a transcription factor, has been shown to bind to the intracellular domain of Notch 1 (Jarriault et al., 1995). Since Notch4 contains the canonical ankyrin/cdc10 repeats, RBP-Jk or RBP-Jk homologues and mammalian Deltex homologues may interact with the cdc10/ankyrin repeats of Notch4. It has been proposed that upon activation of the Notch receptors, Su(H) or RBP-Jk are activated and translocate to the nucleus. where they may regulate transcription of target genes (Goodbourn, 1995). In fact, activated Notch proteins containing only the intracellular domain have been reported to localize to the nucleus (Kopan et al., 1994; Struhl et al., 1993), suggesting a nuclear function for this domain. We have found that the int-3 oncoprotein, modified to encode a flu epitope-tag at the C terminus, is also localized to the nucleus when expressed in cultured 293T cells, as determined by immunofluorescence (unpublished data). The activated int-3 protein lacks a signal peptide but contains a membrane-spanning domain and thus is not likely to enter the secretory pathway. This finding may indicate that int-3 can bind to cytoplasmic proteins that are then translocated to the nucleus.

We show that the *int-3* gene encodes a truncated Notch4 protein with the extracellular domain deleted (EGF-like repeats and Notch/lin-12 repeats), providing the first comparison of a naturally activated murine Notch protein and its normal counterpart. In MMTV-induced mouse mammary tumors with an activated Notch4, as described by Robbins et al. (1992), the oncogenic affects are probably the result of both overexpression or ectopic expression of *Notch4* mRNA as well as functional activation of the Notch4 protein. A structural comparison of the mutant int-3 protein to the normal Notch4 protein is reminiscent of the structural alterations reported to activate the effector function of *Drosophila* Notch and *C. elegans* lin-12 proteins (Greenwald, 1994) or oncogenic activation of TAN-1. Thus, loss of the extracellular domain is likely to lead to loss of the regulatory controls provided by the ligand-binding domain believed to reside in the EGF-like repeats of Notch4.

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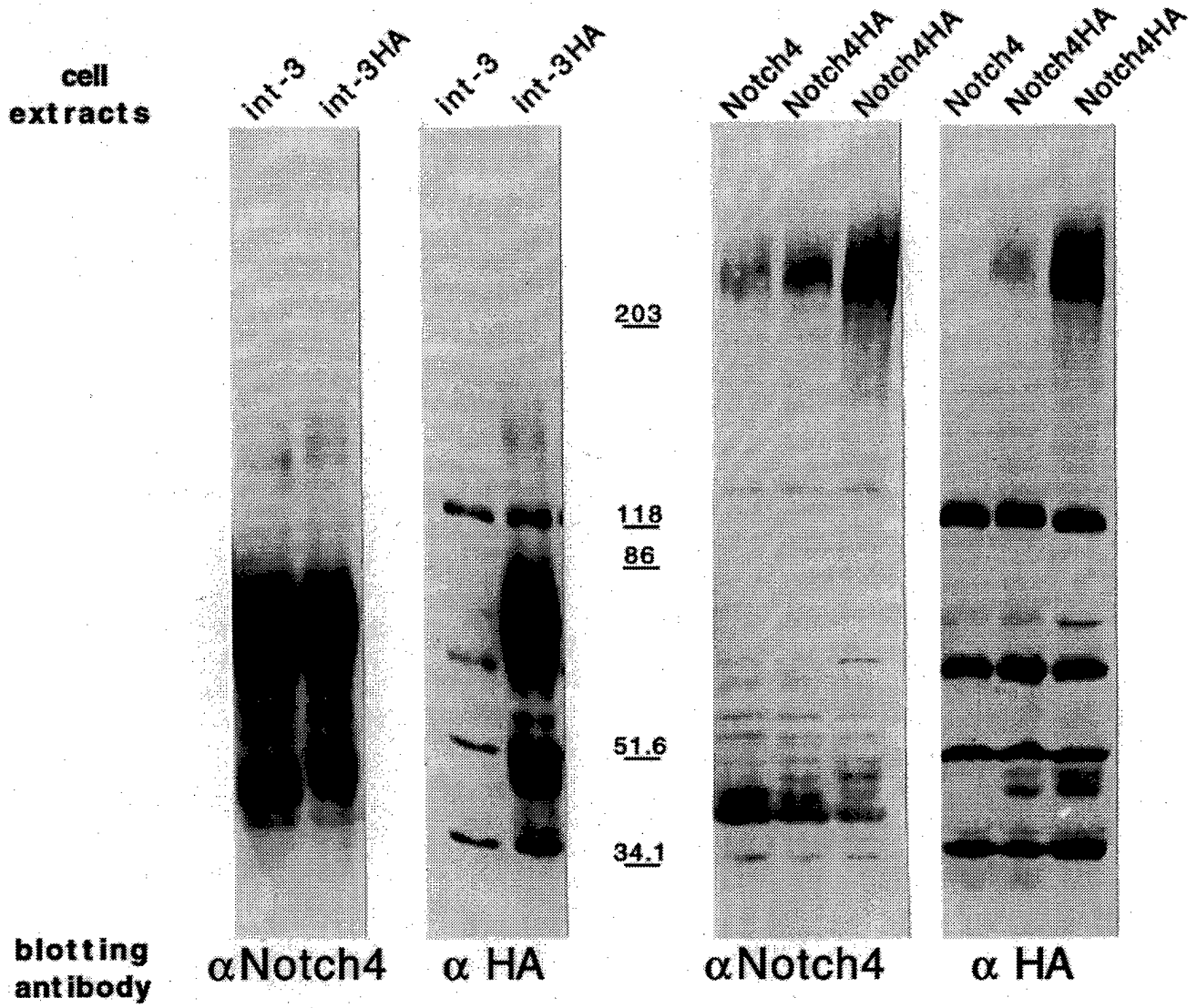
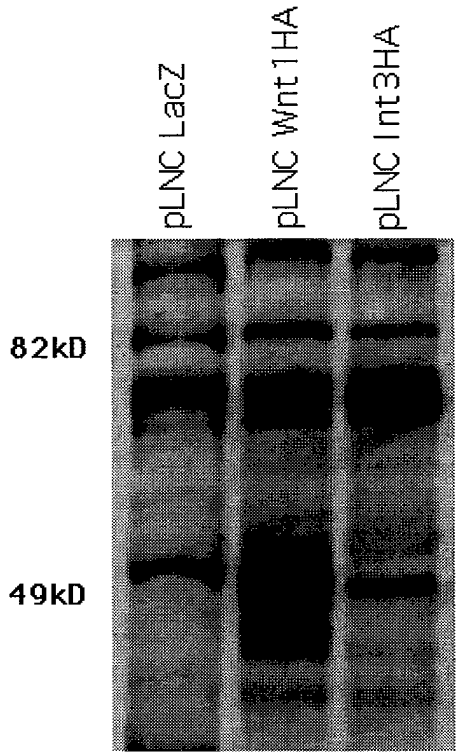
Appendix B

Immunoblot analysis of ectopically expressed int-3 and Notch4 proteins



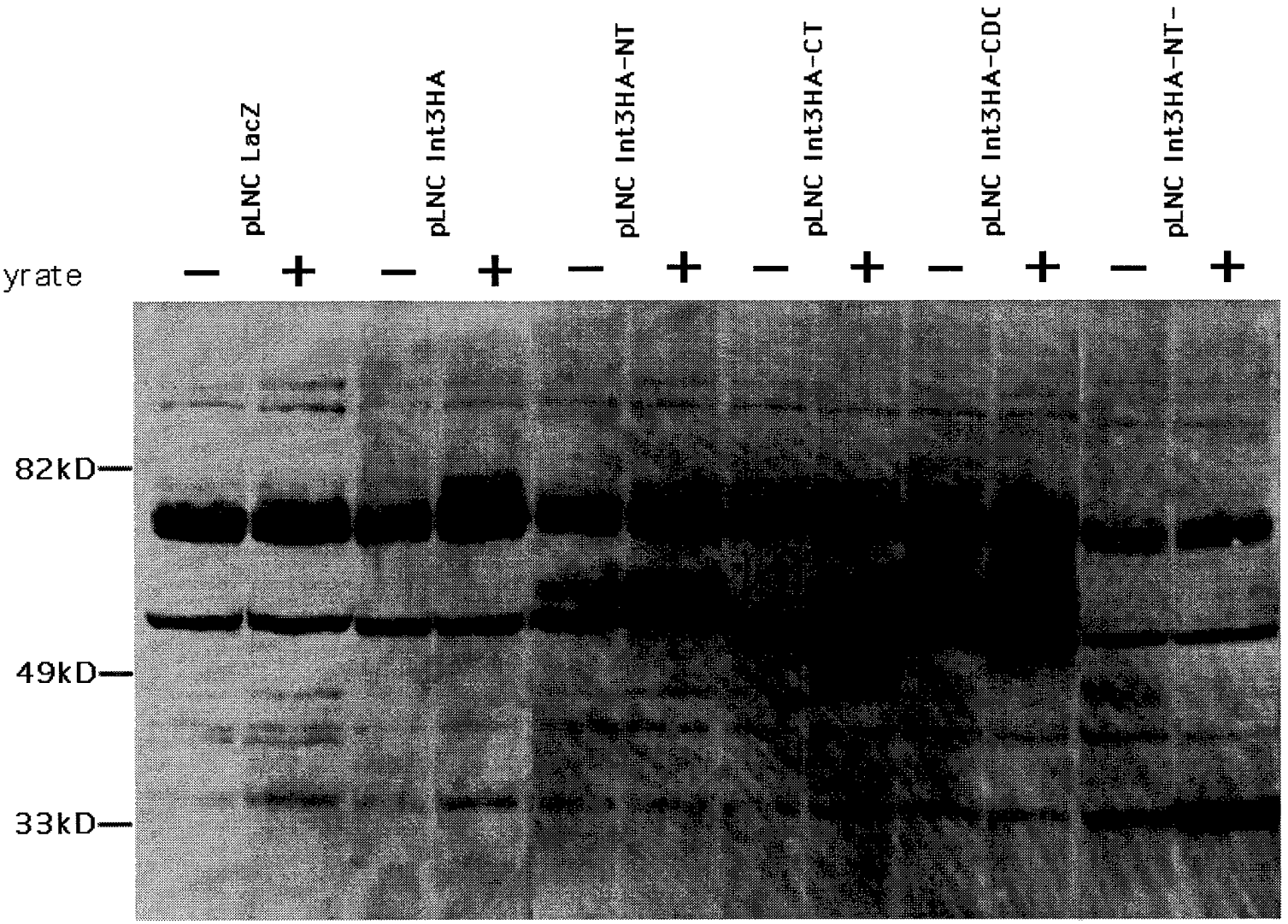
Appendix C



Immunoblot Analysis on Tac-2 protein lysates

50 ug total protein per lane
1 Ab anti-HA 1:50
2 Ab anti-mouse 1:5000
Detection ECL 4 min

Appendix D

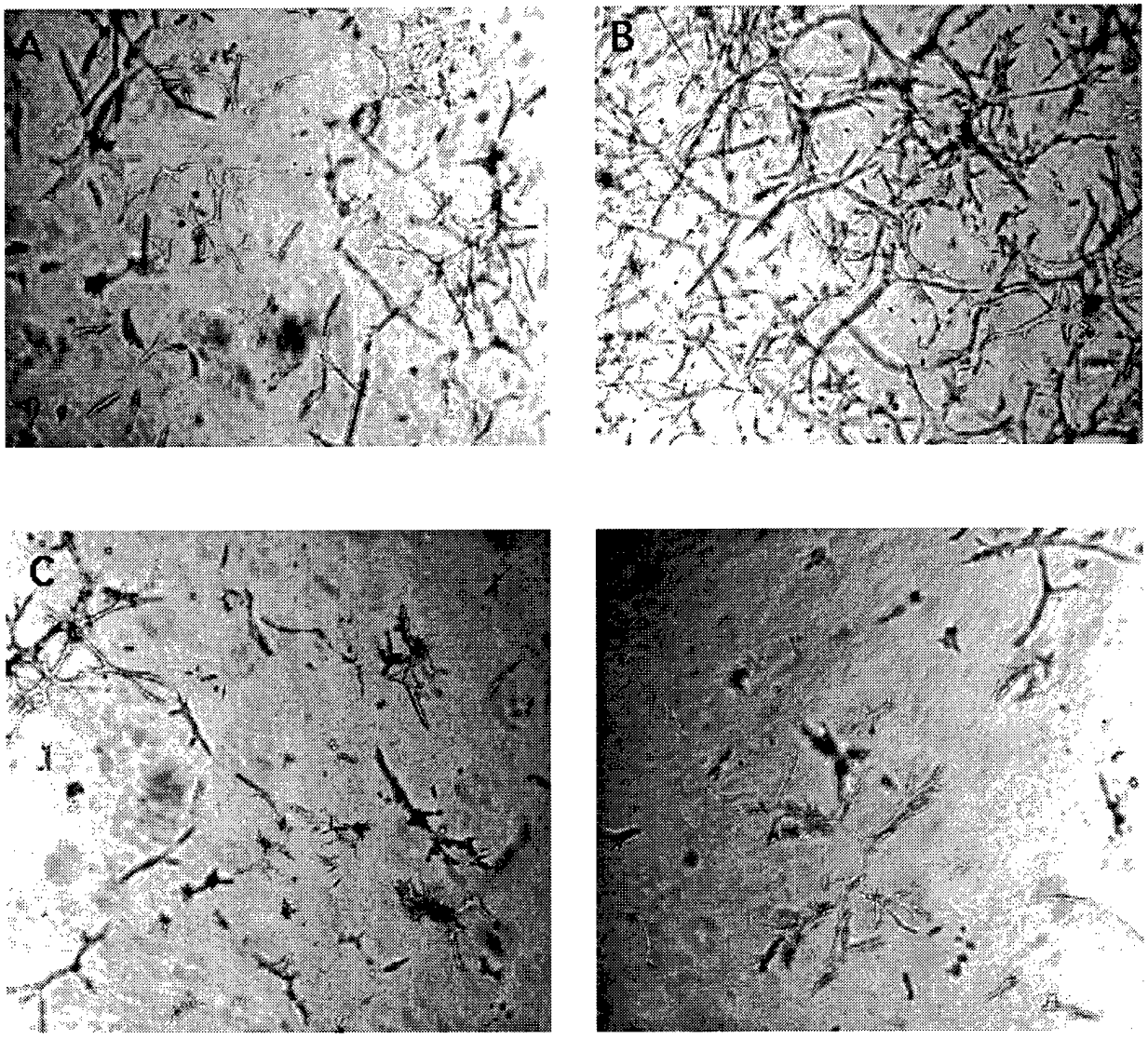


Immunoblot Analysis on Tac-2 protein lysates

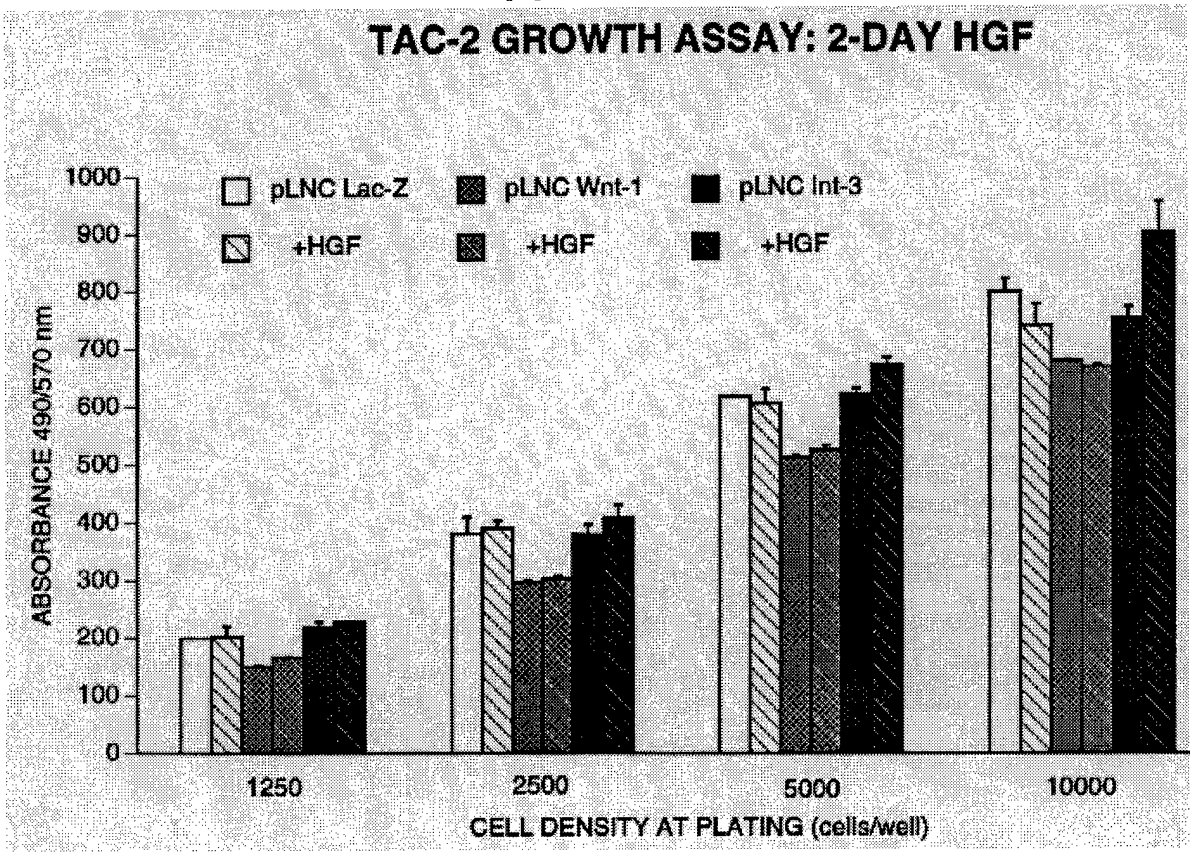
85ug total protein per lane
1 Ab anti-HA 1:50
2 Ab anti-mouse 1-5000
Detection ECL 1 min

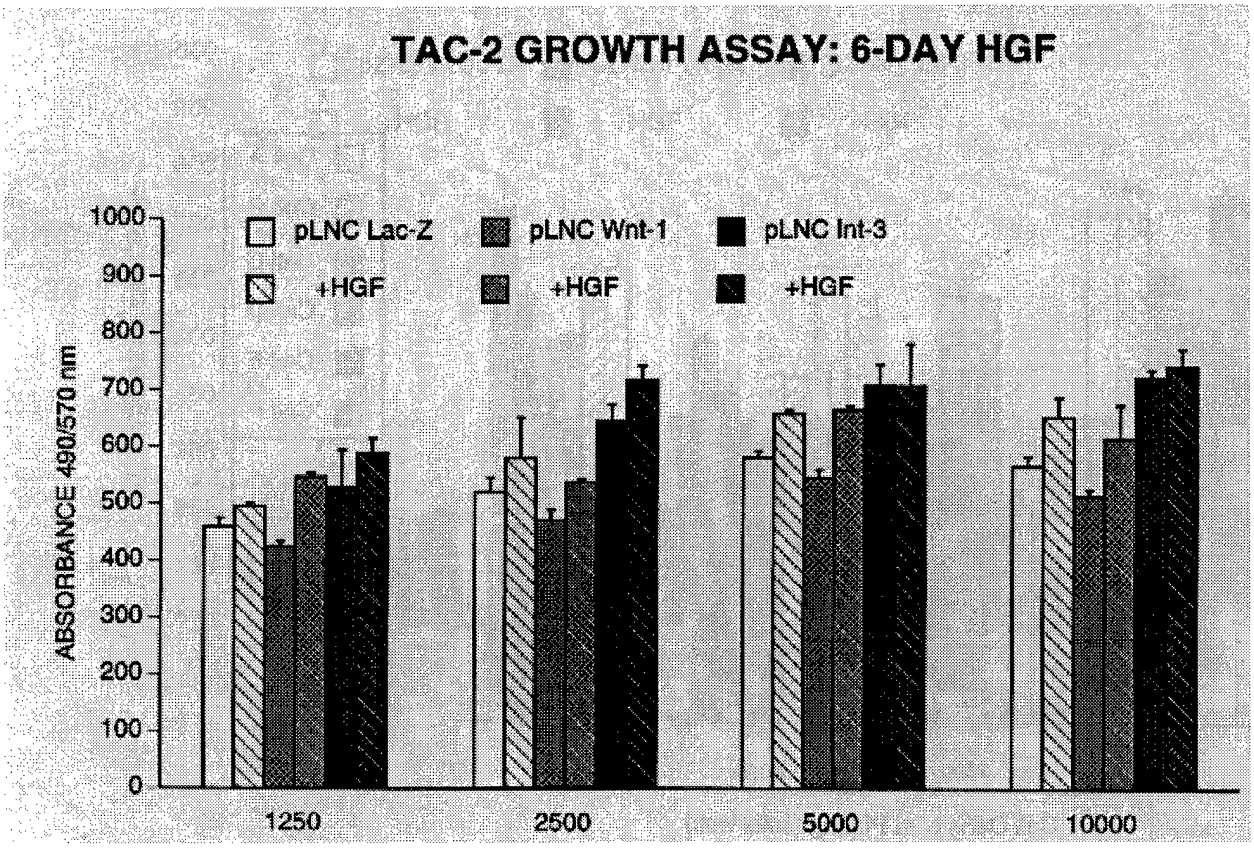
Appendix E

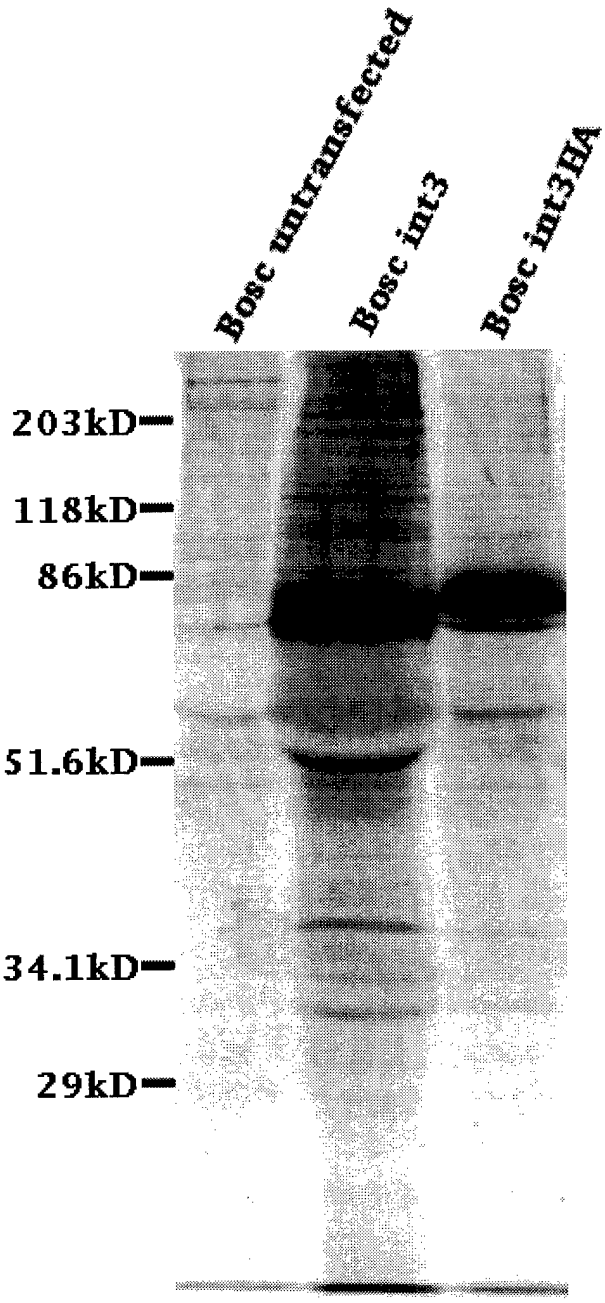
HGF induced Branching Morphogenesis of TAC-2 cells



A TAC-2 LacZ
B TAC-2 LacZ + HGF
C TAC-2 Int3
D TAC-2 Int-3 + HGF


TAC-2 GROWTH ASSAY: 2-DAY HGF
pLNC Lac-Z
+HGF
pLNC Wnt-1
+HGF
pLNC Int-3
+HGF
ABSORBANCE 490/570 nm
1000
900
800
700
600
500
400
300
200
100
0
1250
2500
5000
10000
CELL DENSITY AT PLATING (cells/well)


TAC-2 GROWTH ASSAY: 6-DAY HGF
pLNC Lac-Z
+HGF
pLNC Wnt-1
+HGF
pLNC Int-3
+HGF
ABSORBANCE 490/570 nm
1000
900
800
700
600
500
400
300
200
100
0
1250
2500
5000
10000
CELL DENSITY AT PLATING (cells/well)



Immunoprecipitation of int-3 proteins using rabbit anti-Notch4 antiserum